Evaluation of folate metabolism in patients with Idiopathic Non-cirrhotic Intra-hepatic Portal Hypertension

A dissertation submitted in partial fulfillment of the requirements for DM (Branch IV, Gastroenterology) examination of the Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in August 2009

Certificate

This is to certify that this dissertation entitled ‘Evaluation of folate metabolism in patients with Idiopathic Non-cirrhotic Intra-hepatic Portal Hypertension’ is a bonafide work done by Dr. K. Madhu in partial fulfillment of rules and regulations for DM (Branch IV-Gastroenterology) examination of the Tamil Nadu Dr. MGR Medical University, to be held in August 2009.

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INTRODUCTION

Idiopathic non-cirrhotic intra-hepatic portal hypertension (NCIPH)[1 2 3] is a term that defines one or more entities characterized by intra-hepatic portal hypertension and good to excellent liver function.(See references inserted on the previous page) This definition encompasses a number of entities including non-cirrhotic portal fibrosis (NCPF) and idiopathic portal hypertension (IPH) and is sometimes difficult to differentiate from well compensated cirrhosis. By definition this excludes causes like extra-hepatic portal vein obstruction and Budd Chiari Syndrome.

The etio-pathogenesis of NCIPH is poorly understood with a number of hypotheses proposed in the past. Arsenic toxicosis from contaminated drinking water is a possible etiological factor for NCIPH in India[6] .Infective hypothesis was put forward with the possibility of umbilical sepsis, bacterial infection and diarrhoeal episodes in infancy and early childhood leading to portal pyemia, pylephlebitis resulting in thrombosis, sclerosis and obstruction of small and medium sized portal vein radicals [7].

The role of prothrombotic disorders in causing obstructive portal venopathy has been investigated. One study from the West showed the prevalence of identified prothrombotic disorders in the study cohort was higher (54%) when compared to that expected in the general western population (8-15%)[8]. A deranged immune response and reduced cell mediated immunity was seen in patients with NCPF in a study published from India[9] .

In this present study we aimed to evaluate the folate metabolism with special reference to methylene tetrahydrofolate reductase(MTHFR) gene mutations in patients with NCIPH. Vitamin B12 and folate are essential cofactors in homocysteine metabolism and deficiency of these vitamins are associated with hyperhomocysteinemia.[26] which is associated with increased risk of arterial and venous thrombosis [27.] A recent study from Turkey which looked at thrombophilic mutation profile in NCIPH showed significantly higher incidence of MTHFR gene mutations in patients than controls [28] . The aim of the present study was to evaluate the serum levels of folate, vitamin B12 and homocysteine and to evaluate methylene tetrahydrofolate reductase (MTHFR) gene mutations in patients with NCIPH spectrum disease and to compare them with cirrhosis of known cause and matched healthy controls.

AIMS & OBJECTIVES

The hypothesis is that non-cirrhotic intrahepatic portal hypertension (NCIPH) occurs secondary to a defect in folate metabolism, that leads to a procoagulant status and vascular occlusion.

The aims of the present study were to evaluate the serum levels of folate, vitamin B12 and homocysteine and to evaluate methylene tetrahydrofolate reductase (MTHFR) gene mutations in patients with NCIPH spectrum disease and to compare them with cirrhosis of known cause and matched healthy controls.

REVIEW OF LITERATURE

Historical background:

During 1984 to 1910, Banti In Italy proposed the disorder morbus Banti, which is characterized by primary cryptogenic splenomegaly and anemia not associated with any known hematologic disease[10].

In 1954, Tisdale et al described four patients with portal hypertension and massive bleeding from esophageal varices in whom no intra hepatic nor extra hepatic portal obstruction was found[11].

Later Ramalingaswami et al in India (in 1962) noticed a similar disease while studying autopsy materials and characterized the histological lesion as obliterative portal venopathy [12]. In 1969, the title “non-cirrhotic portal fibrosis’ was officially adopted at a workshop organized by the Indian Council of Medical Research.

Mikkelsen et al in Los angeles described 35 patients with splenomegaly and non-cirrhotic portal hypertension in whom phlebosclerotic processes were apparent in the intra and extra hepatic portal vein system and called the disease as “ hepato portal sclerosis” [13]

With similar publications coming from various countries it is established that this entity called with different names does exist throughout the world, being more common in developing countries. The various names that are synonymously used are Hepatoportal sclerosis, NCPF, Obliterative portal venopathy, Non cirrhotic intra hepatic portal hypertension and Idiopathic presinusoidal portal hypertension.

Epidemiology:

NCIPH has been reported to be common in socioeconomically disadvantaged in India.

In 1985, the reported incidence of IPH in Japan was $0.75/10^5$population and in 1992 only an average of 11 new patients were reported showing a declining incidence (related to improved hygiene and standards of living)[14]. The incidence of IPH has not been prospectively studied in India. Most of the services from difference part of India show a male predominance of 2: 1 to 4:1[12,15,16].

In Japan, IPH was more common in older females with a female to male ratio of 3:1 and an average age of 40.6 years[12].

In a study from North India it was reported that NCPF is on decline in India[18]. But a recent study from our centre documented NCIPH as a common cause of cryptogenic intra hepatic portal hypertension (to be published).

Etiology:

Etiopathogenesis of NCIPH is poorly understood and a number of hypotheses have been proposed.

Infective hypothesis:

Abdominal infection at birth or in early childhood has been alleged to play an important role[19]. Umbilical sepsis, bacterial infections and diarrheal episodes in infancy and in early childhood are likely to lead to portal pyemia, pylephlebitis, resulting in thrombosis,sclerosis and obstruction of small and medium sized portal vein radicals[29].

Exposure to Chemicals:

Prolonged ingestion of arsenic has been related to causation of NCPF[20]. Histological picture that resembles NCPF has been observed following chronic exposure to vinyl chloride monomers, copper suflate (vineyard sprayers), protracted treatment with methotrexate, hypervitaminosis A and in recipients of renal allografts who received treatment with 6-mercaptopurine, azathioprine [21].

Immunological and immunogenetic hypotheses:

Evidence supporting these hypotheses includes: a reduction in the suppressor/cytotoxic T lymphocytes (T8) in NCPF patients

i. a decreased T4/T8 lymphocytic ratio

ii. a reduction in the cell-mediated immune status in NCPF patients

iii. poor autologous mixed lymphocyte reaction (MLR)[22]

No definite genetic predisposition to NCPF has been reported.

Prothrombotic disorder:

In a retrospective study of 28 NCIPH patients, prothrombotic disorders were detected in 12 (48%) patients[8]. The disorders found were myeloproliferative disorder, protein C and S deficiency and APL syndrome.

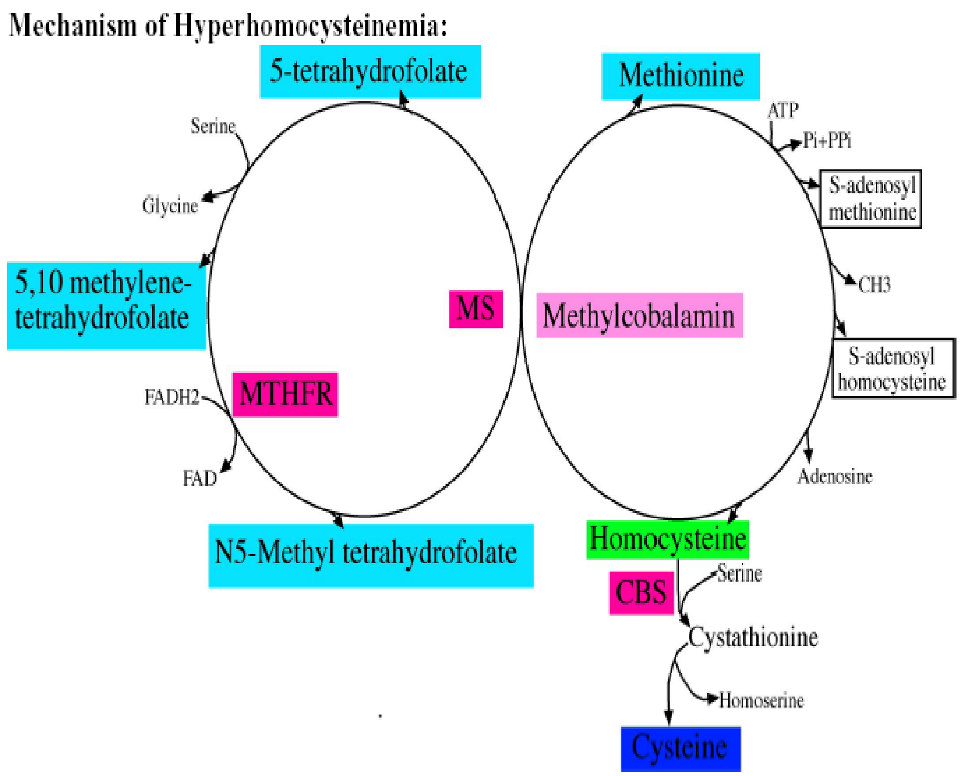
There were two case reports of NRH(Nodular regenerative hyperplasia) associated with IgA aCL and evidence of poorly compliant coeliac disease[54]. There was a suggestion that T cell help from gluten specific T cells is responsible for driving the IgA autoantibody response to both transglutaminase and protein/phospholipid complexes, leading to the formation of IgA aCL. IgA aCL then trigger thrombosis in small portal vein radicles, which drain the inflamed small intestine.

Hyperhomocysteinemia – Prothrombotic state

Homocysteine is a naturally occurring molecule in the body and it is required in several reaction within the cells resulting in the formation of cysteine and methionine, which can be in the further used by the body. If the pathways to either cysteine or methionine are blocked, then homocysteine levels may use.Methionine synthase requires vitamin B12 (methylcobalamin) in order to carry out its reaction. If a patient does not have an adequate supply of vitamin B12, then homocysteine is not converted to methionine and the net result is an increase in homocysteine. MTHFR is required to form 5-methyltetrahydrofolate which is required to convert homocysteine to methionine. If this cannot be formed, then homocysteine levels will increase. Cystathionine betasynthase is required to convert Homocysteine to methionine and its deficiency also leads to increased homocysteine.

BACKGROUND ON FOLATE AND B12 METABOLISM

Folic acid is a carrier of one-carbon fragments which it transfers to various biochemical targets. The one-carbon piece can be in several different oridation states but two important forms of folic acid are methyltetrahydrofolate and methyelene –THF



Three factors involved in the methionine cycle could influence plasma homocysteine concentrations: MTHFR polymorphism, vitamin B12- an essential cofactor and folate- the substrate.

Methyelene tetrahydrofolate reductase 677 (MTHFR 677) polymorphism ay provide hyperhomoysteinemia when folate status is low.

In a study published from Germany[23] it was found that MTHFR 677 TT individuals are more liable to hyperhomocysteinemia under vitamin B12 deficiency than the other two genotypes. In such a case, relative folate shortage may progressively increase homocysteine levels. TT individual may have increased folate and vitamin B12, requirements compared to the other CC and CT genotypes[23].

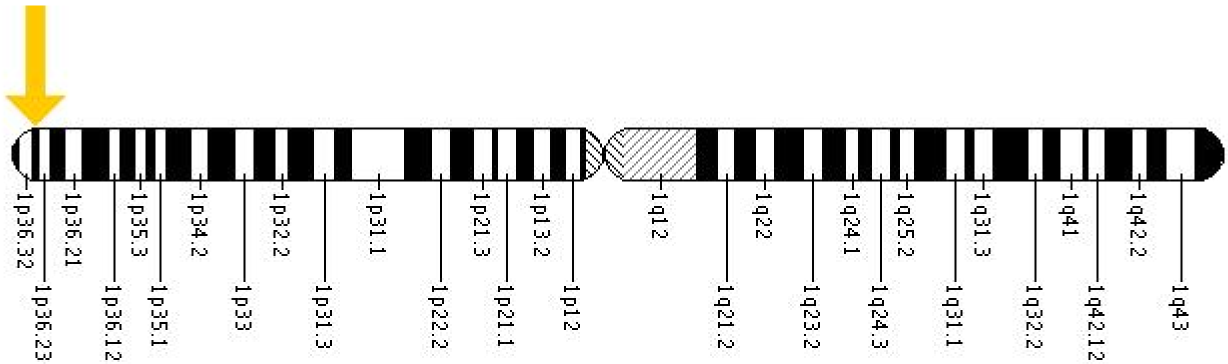
An Elevated Homocysteine level

"Normal" and "abnormal" values are set by individual laboratory. Typically, a level less than 13 micr mol/L is considered normal. A level between 13 and 60 u mol/L is considered moderately elevated. A value greater than 60-100 u mol/L is severely elevated[24]

Risks associated with Elevated homocysteine levels[25]:

- Coronary artery disease (atherosclerosis)
- Heart attack
- Stroke
- Peripheral arterial disease
- Venous thrombosis – Deep vein thrombosis, Pulmonary embolism
- Dementia
- Having a child with a neural tube defect.

The 5,10 MTHFR gene is located on chromosome at 1 at 1p36.3. The complementary DNA sequence is 2.2 Kilobases long and consists of 11 exons[30].



The major product of the MTHFR gene is a catalytically active 77 Kilodalton protein .

5,10-methylene tetrhydrofolate reductase (MTHFR) converts 5,10 methylene THF into 5-methyl THF, a major circulating form of folate.

5 methyl THF provides methyl group for homocysteine methylation[31]. Two common polymorphism in the MTHFR gene, a C to T transition at nucleotide 677 (C677T) ,an A to C transition at portion 1298 (A1298C) are associated with a 50-60% decrease in catalytic activity[33].

The T677 variation causing alanine to valine substitution at the codon 222 affects the catalyte MTHFR domain leading toa thermolabile enzyme with reduced catalytic activity. Homozygosity for valine 222 predisposes to the development of hyperhomocysteinemia, especially during times of folate insufficiency.

A C 1298 variation causing the replacement of glumate by alanine at the codon position 429 affects regulatory MTHFR domain. Homozygosity for alanine 429 is associated with lower enzymatic activity but does not seem to influence homocysteine plasma levels, except when accompanied by T677 mutation[33].

C677 T allele: This is characterized by a point mutation at position 677 of the MTHFR gene that converts a cytosine C into a thymineT. This mutation results in a amino acid substitution (alanine to valine) in the enzyme. The C677T allele is commonly called "thermolabile", because the activity of the encoded enzyme is reduced at 37^0 C or more.

A1298 allele (A1289 C) – In the A1298 C allele, a point mutation in exon 7 results in an amino acid substitution (glutamate for alanine) in the enzyme. This allele has also been called the C1289 A allele. The activity of the encoded enzyme is decreased, although less than is the case with the C677T allele. People who are homozygous for the A1298C allele do not appear to have high serum homocysteine levels than controls. However, people who are compared heterozygous for the A1298C and C677T alleles tend to have a biochemical profile similar to that seen among C677T homozygotes, with increased serum homocysteine levels and decreased serum folate levels[33].

Population Frequencies:

In a study from South India. homozygous MTHFR A1298C SNP was detected in 15.3% of the individuals tested and 47.2% were heterozygous for this SNP.

Homozygosity for the C677T SNP (single nucleotide polymorphism) was detected in 1.38% and the frequency of the C677T heterozygotes was 18.1%. The frequency of double heterozygosity was 19.6% and the frequency of double homozygosity was completely absent[34].

MTHFR and Liver Disease

In an Italian study [35] ,MTHFR C677T mutations were looked at in patients with liver cirrhosis (LC) and with or without portal vein thrombosis (PVT). Multivariate analysis of seven variables showed a statistically significant relationship between MTHFR C677T homozygous state and cryptogenic liver cirrhosis(P value of 0.004 ; Odds ratio of 6.87; 95% CI 1.43–25.55),age ,alcohol abuse and presence of PVT. The conclusion was that MTHFR C677T-related endothelial damage can result in both PVT and vascular damage, ischemic injury and fibrosis, prompting cirrhosis development from any etiology.

In a study from Turkey which analysed thrombophilic mutations and natural anticoagulant deficiency in patients with Idiopathic portal hypertension, MTHRF C677T and MTHFR A1298C frequencies of genetic polymorphisms were found to be significantly higher among patients than that of controls[36].

Clinical presentation of NCIPH:

These patients present with well tolerated episodes of gastrointestinal haemorrhage, a long standing mass in the left upper quadrant (splenomegaly), anemia and consequences of hypersplenism.

Development of ascites, jaundice and hepatic encephalopathy is uncommon and may be seen only after an episode of gastrointestinal haemorrhage. Of all the causes of portal hypertension, a massive and disproportionately large spleen is seen most commonly in NCPF. Left upper quadrant pain due to perisplenitis and splenic infarction is not uncommon. NCPF also may have odd presentations, such a glomerulonephritis, hypoxemia or autonomic dysfunction[37].

Major presenting symptoms in Japanese patients with IPH were splenomegaly (88%), hepatomegaly (44%), gastrointestinal bleeding (35%) and ascites (12%).

Natural history of NCPF/IPH

The survival curve for patients with NCPF/IPH is somewhat between that for those with cirrhosis and for a healthy population of comparable age[39]. Good prognostic features in patients with NCPF, a 2- and 5-year survival of nearly 100% after successful eradication of esophagogastric varices, have been described [29]. Hillaire et al [8] reported death in 4 out of 28 patients with IPH owing to terminal liver failure. Development of PVT in a patient with IPH may be a significant factor for poor prognosis, and ascites may indicate a deterioration of the condition in patients with IPH. Furthermore, PVT and ascites may be mutually related in this disease.

Histopathology of NCPF/IPH

Autopsy liver- Gross examination may reveal a normal, enlarged, or even shrunken liver. Subcapsular septation can be noted, while deeper parenchyma shows normal architecture.Sclerosis of large to small intrahepatic portal vein branches and approximation of portal tracts to surface has been documented [39].Histological features noted in autopsies include increased portal collagenous connective tissue and sclerosis and obliteration of small branches of portal veins in most cases[4]. This histological hallmark of NCPF was termed *obliterative portal venopathy* by Nayak and Ramalingaswami. Intimal fibrosis and elastosis is also common, leading to subendothelial thickening of the wall of large- and medium-sized portal vein branches even compromising the lumen. Veins may be thickened to the extent that they resemble an artery. Furthermore, aberrant vasculatures characterized by thin-walled vessels mainly located adjacent to the portal tracts and at times in the hepatic lobules have been described. Mild inflammation is seen in a few cases. Incomplete portal pseudolobule and scattered regenerative nodules may be noted in a few cases.

Needle biopsies-Biopsy material may show only mild and subtle changes from normal. These changes include inconspicuous portal tracts and obliterated veins, or fibrous expansion of portal tracts[4]. Alternatively there may be dilatation of vessels in or near portal tracts, with vessel-like dilatation of sinusoids. Ludwig et al. studied the changes in 25 liver biopsies. Changes in the portal tract included capillary dilatation, phlebosclerosis, and fibroelastosis of the stroma. Portal vein dilatation with herniation into the surrounding hepatic parenchyma was also noted[35].

Wedge biopsies- Wedge biopsies show changes similar to autopsy material, but changes in medium and large portal vein branches may not be seen if not sampled[4]. A deep-core wedge biopsy (not broad-based wedge) along with a needle biopsy should be taken, as they would complement each other in the information provided. This material may be valuable in looking for clues to the etiopathogenesis of NCPF

Laboratory features:

Patients usually have preserved hepatic function.The tests of liver function are normal. Pancytopenia is found in the majority of patients. Anemia may be microcytic, hypochromic (due to GI blood loss) or normocytic, normochromic (due to hypersplenism)[4]. Leucopenia (<4000/cumm) and thrombocytopenia (platelets <50,000/cumm) may also be present due to hypersplenism. Whether the leucopenia in NCPF increases susceptibility to infections and whether splenectomy is required in such cases remain debatable. The bone marrow is hypercellular. Coagulation and platelet function anomalies also have been observed. Mild compensated disseminated intravascular coagulation secondary to endotoxemia or portosystemic collaterals has been reported in a fair proportion of these patients[41].

Imaging:

Ultrasonography shows a dilated and patent splenoportal axis with significantly thickened walls of the portal vein and to main branches. Doppler studies are helpful in identifying an occasional patient who has a thrombus in the intrahepatic branch of the portal vein[42].

Endoscopy:

Esophagogastric varices are seen in 85-95% of patients who have NCPF. Furthermore, patients with NCIPH have large varices more often (90%) compared with cirrhotic patients (79%).Anorectal varices also are more common (90% versus 56%) and are bigger in size[53].Gastric varices are more common in NCPF.

Hemodynamics in NCPF/IPH:

The portal vein pressures are elevated markedly in patients who have NCPF. Two pathoanatomic sites of obstruction have been identified. A pressure gradient exists between the spleen and the liver (intrasplenic pressure – intrahepatic pressure [IHP]) and another exists between the IHP and the wedged hepatic venous pressure(WHVP) (IHP – WHVP). Generally, the WHVP is normal or only slightly elevated in NCPF. Variceal pressure also has been studied in these patients and is comparable to that in cirrhotic portal hypertension[44].Intravariceal pressure closely reflects portal pressure in patients who have NCPF and is the investigation of choice for measurement of portal pressure.

Management:

For acute bleeding varices, variceal band ligation and endoscopic sclerotherapy are equally efficacious (approximately 95% success in control of acute bleed)[45].Prevention of re-bleeding with the use of non-selective β-blockers has been reported. B-blockers and endoscopic variceal ligation was found to be equally efficacious for primary prophylaxis in non-cirrhotic patients[46]. Gastric varices, can be managed with cyanoacrylate glue injection and rarely require surgical intervention. Interventional radiological procedures have been reported to be effective in patients with IPH. These include splenic embolisation, percutaneous transhepatic obliteration and a transjugular intrahepatic porbosystemic shunt (TIPSS) procedure.

The role of surgery is limited in variceal bleed because it is required in less than 5% of cases of NCPF that fail to respond to endoscopic therapy[47]. Selective shunts like distal splenorenal shunts are preferred because they have a lower incidence of post shunt encephalopathy. However if massive splenomegaly is present, the proximal shunt with splenectomy is a good choice. Surgery also is indicated for patients who have symptomatic hypersplenism, for patients who hail from distant areas, or for those who desire one-time treatment. The surgical mortality after emergency shunts is about 10%. Shunt occlusion, overt chronic porto systemic encephalopathy and re-bleeding after elective shunt surgery are seen in about 10% patients[45]. After successful eradication of esophago-gastric varices, 100% 2 and 5 year survival is observed in these, patients[48]. The long term outcome of shunt surgery also is favorable and 88% 5 year survival has been reported.

METHODOLOGY

STUDY DESIGN:

CASE CONTROL STUDY-

Folate metabolism was prospectively analysed in patients with Idiopathic noncirrhotic intrahepatic portal hypertension(NCIPH) and compared with age and geography matched healthy controls and disease controls

Period of recruitment was over a period of 2 years (JAN 2007 to JAN 2009). Blood samples for evaluation of folate metabolism including MTHFR gene mutation analysis were collected in the Liver clinic OPD or in the WARD.

The study was approved by Research and Ethics committees (IRB) of the Christian Medical College, Vellore.

Inclusion Criteria

1. **Subjects with NCIPH :**

   a. Age 18 – 70 years

   b. Evidence of portal hypertension – esophageal varices, hypersplenism, ascites

   c. Doppler ultrasound – showing patent portal and hepatic veins

   d. Liver biopsy showing no cirrhosis

e. Exclusion of conditions causing cirrhosis, such as chronic viral hepatitis, alcoholic liver disease, non-alcoholic steatohepatitis (NASH), obesity, hemochromatosis, autoimmune hepatitis, Wilson's disease, chronic vitamin A intake, professional exposure to copper sulphate, vinylchloride, past angiography with thorium sulphate, exposure to arsenic salt/herbal medicine.

2. Disease Controls:

a. Age 18-70 years

b. Cirrhosis of known aetiology – HBV, HCV

3. Healthy Controls – Age, Sex and Geography matched.

Variables: The following variables were measured:

- Serum vitamin B12,
- Serum folate
- Fasting serum homocysteine
- MTHFR gene mutation analysis

METHODS:

Serum Vitamin B12 – was measured by competitive chemiluminescent enzyme immun**oas**say involving an automated alkaline denaturation procedure using IMMULITE 2000 analyzer(DPC California). Serum levels less than 200 pg/ml were considered to be low ,but for the purpose of the study we took levels less than 250 pg/ml to be low. This is because almost all the patients attending the liver clinic had been to various hospitals before and would have received vitamin supplements .

Serum Folate – was measured by competitive immunoassay using IMMULITE 2000 analyzer and levels less than 3 ng/ml were considered to be low.

Fasting serum homocysteine levels – were measured by HPLC (High performance liquid chromatography) .Typically, a level less than 13 micro mol/L is considered normal.A level between 13 and 60 u mol/L is considered moderately elevated. A value greater than 60-100 u mol/L is severely elevated[19]

Analysis of MTHFR gene mutation – PCR(Polymerase chain reaction) / RFLP(Restriction fragment length polymorphism)

GENETIC ANALYSIS

DNA isolation:

Blood samples were collected in EDTA coated vacutainer tubes Greiner bio-one) from individuals with NCIPH, healthy controls and patients of HBV/HCV related cirrhosis after getting informed written consent. DNA isolation was carried out by salting-out method . This method was chosen because this method replaced corrosive organic solvents used in the conventional method with saturated salt solution and hence non hazardous to the user and the environment.

Blood samples were centrifuged at 4000 rpm for 15 min at 4 °C to collect the WBC rich buffy coat. This step was used to reduce the usage of RBC lysis buffer volume and also to improve the DNA yield. Three layers were visible after centrifugation and they were: the top yellow layer containing the plasma, the middle white layer was the buffy coat and the bottom red was the erythrocytes. Plasma was carefully removed and discarded in a disposal glass beaker and then the buffy coat was collected along with little of erythrocytes in a fresh tube. Ten volumes of RBC lysis buffer was added and incubated at room temperature for 10 min. The lysis was checked by looking at the transparency of the solution. Then the tube was centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was discarded in a disposal glass beaker leaving behind the white pellet of WBC in the tube. The wash was repeated until a clear white pellet was obtained. To the pellet 4.5 ml of WBC lysis buffer was added and the pellet was disturbed to get homogeneous solution. 250 µl of 10% SDS and 100 µl of Proteinase K was added and mixed well and incubated at 37° C for overnight or at 55°C for two hours. The lysis was checked at the end of the incubation period and in the case of incomplete lysis, 50 µl of Proteinase K was added and the digestion period was extended. After the digestion, 1.5 ml of saturated NaCl solution was added and mixed well till the solution turned milky white. This was then centrifuged at 4000 rpm for 15 min at 4°C and the supernatant was collected in a fresh 15 ml centrifugal tube. Two volumes of absolute ethanol was added to the supernatant and mixed gently, a white thread like structure appeared and reached the top of the solution. The thread like structure was collected in a fresh eppendorf tube containing 1ml of 70 % ethanol, using a sterile pipet tip. The tube was tapped to remove the excess salt adhering to the precipitate, and then the precipitate was transferred into another sterile eppendorf tube and allowed for air dying. When the precipitate turned transparent, 200-300 μl of TE buffer was added and stored in -20°C deep freezer until the polymorphic analysis was carried out.

MTHFR POLYMORPHIC ANALYSIS

The two MTHFR gene polymorphisms, C677T and A1298C were analyzed by PCR-restriction fragment length polymorphism (PCR-RFLP) method . The primer sequences were: 5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and 5'-AGG ACG GTG CGG TGA GAG TG-3' for C677T and 5'-CTT CTA CCT GAA GAG CAA GTC-3' and 5'-CAT GTC CAC AGC ATG GAG-3' for A1298C.

The PCR reactions were carried out in 20 μl volume and each reaction mix contained 1x buffer with 1.5 mM $MgCl_2$, 200 μM of dNTPs (Finnzymes, Finland), 250 nM of forward and reverse primers (Sigma genosys, India), 1 unit of JumpStart Taq DNA polymerase (Sigma, USA). The PCR condition was optimized by performing gradient PCR with a temperature range of 55-65°C. The thermal cycling protocol for C677T and A1298C polymorphisms comprised of initial denaturation at 95°C for 1 min, cycle denaturation at 94°C for 30 sec, annealing temperature at 63°C for 30 sec, extension at 72°C for 30 sec, and the cycle was repeated for 39 more times, final extension at 72°C for 8 min. The PCR products were checked for amplification by resolving on 2% agarose gel electrophoresis and checked with UV transilluminator (Vilbert Lourmat, France).

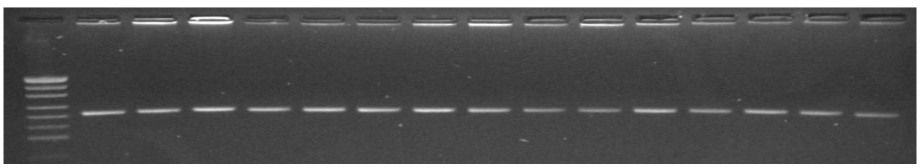
The PCR products sizes were 198 and 256 bp respectively for C677T and A1298C.Then the amplified samples were digested with 1 unit of restriction enzymes, HinfI and MboII (New England Biolabs) respectively for C677T and A1298C analysis at 37°C for 16 hours. The digested PCR products were resolved on 2% agarose gel electrophoresis and the gel patterns were documented using a gel documentation system (Vilbert Lourmat, France). In the C677T analysis, the digestion pattern of two bands of size 175 and 23resulted from complete digestion corresponds to mutant

TT genotype and three bands with 198, 175 and 23 bp corresponds to CT genotype whereas the undigested marked the presence of CC genotype. The complete digestion resulting in four bands of 176, 30, 22 and 28 bp represents wild AA genotype; partial digestion resulting in five bands of 204, 176, 30, 22 and 28 bp corresponds to AC genotype whereas digestion yielding 204, 30 and 22 bp showed the presence of CC genotype in A1298C analysis.

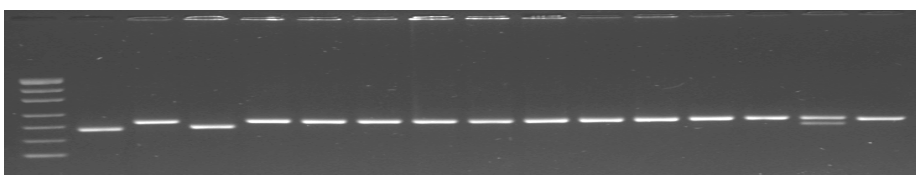
Polymorphism analysis of the methylenetetrahydrofolate reductase gene (MTHFR) amplicons by agarose gel electrophoresis after restriction endonuclease digestion (RFLP). – ¨677 position was digested by HinfI revealing the genotypes CC(wild type)(198bp band), CT and TT (175bp band). B) – 1298 position was digested by MboII and presented the genotypes AA (wild type, 100bp band), AC and CC (72bp band)

C677T POLYMORPHISM

PCR AMPLIFICATION

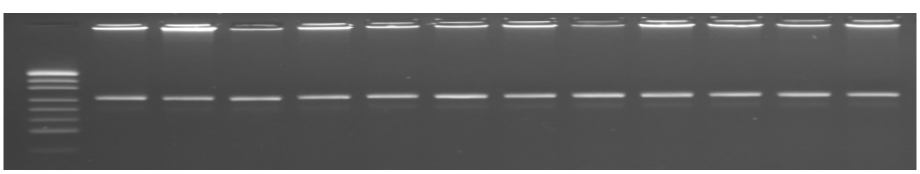


RESTRICTION FRAGMENT LENGTH POLYMORPHISM

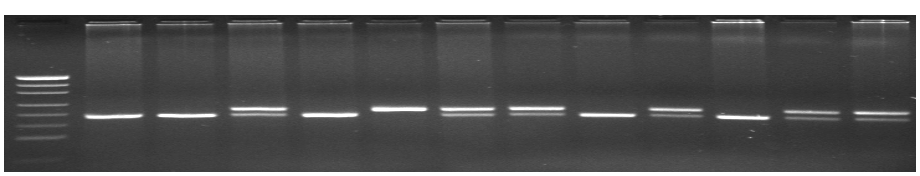


A1298C POLYMORPHISM

PCR AMPLIFICATION



RESTRICTION FRAGMENT LENGTH POLYMORPHISM



CONSENT:

Informed written consent (Annexure 1) was taken for blood collection and DNA analysis prior to the study. Peripheral blood sample (10ml) was collected from the antecubital vein using EDTA coated vacutainer tubes. This blood was stored at -20 degree Celsius. Genetic studies were performed at Wellcome Research Laboratory ,CMC,Vellore .

STATISTICAL ANALYSIS:

Data was analysed by statistical software SPSS (Statistical Package for Social Sciences, release 11.0, standard version; SPSS Inc).

This was a prospective case control study and data of the cases(NCIPH) was reported as median with ranges for continuous variables and as frequencies and percentages for categorical variables.

Fisher exact test was used to assess for significance of genetic mutations in cases and controls. A **p** value of less than or equal to 0.05 was considered statistically significant. Spearman's correlation was used to calculate the correlation coefficient between vit B12 and serum homocysteine, MELD score and vit B12 in NCIPH patients. Simlarly correlation coefficient was calculated between MELD and vit B12 in cirrhotic patients.

RESULTS

Age Distribution:

The median age of presentation in NCIPH patients was 32.5 years, the youngest being 15 years and eldest being 62 years.

Sex distribution:

31 (68%) of the patients were male and the rest 15(32%) were female, the male to female ratio being 2.13:1

Geographic distribution:

20 patients (43%) were from Eastern India

16 patients(35%) were from South India

10 patients (22%) were from Northern India

TABLE 1 -BASELINE CHARECTERISTICS OF NCIPH PATIENTS (N=46)

VARIABLE	MEDIAN (RANGE)
AGE (yrs)	32.5(15-62)
SEX*	M-68%
Hb(gm%)	9.3(3.8-15.1)
TC(/cmm)	3000(900-11600)
PLC(/cmm)	54000(19000-490000)
T.BILIRUBIN(gm/dl)	1.35(0.5-6.1)
T.PROTEIN(gm/dl)	7.5(5.2-9.0)
ALBUMIN(gm/dl)	3.8(2.4-4.6)
AST(U/L)	42.5(18-77)
ALT(U/L)	28.5(12-96)
ALKALINE PHOSPHATASE	100.5(35-72)
MELD	9(6-16)
HVPG(mmHg)	7(2-18)
VIT B12(pg/ml)	323(50-1976)
FOLATE(ng/ml)	14.3(1-22.6)
SERUM HOMOCYSTEINE(micromoles/L)#	15.2(4.5-29.5)

#-measured in 19 NCIPH patients

* Percentage of total NCIPH patients

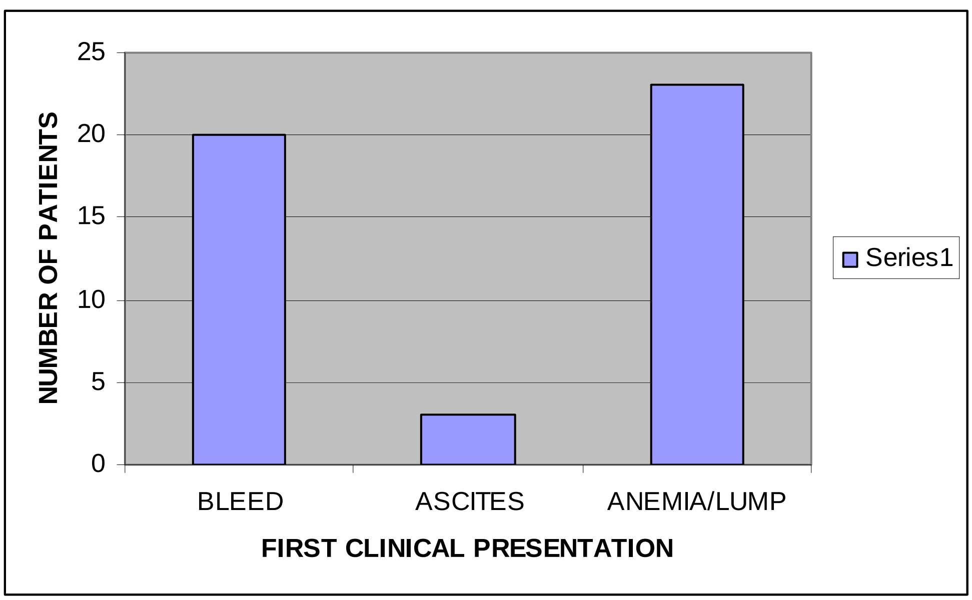
Clinical presentation and lab parameters of NCIPH patients:

Anemia / lump in the abdomen was the first presentation in 50%(23) of the patients of which 17 patients first visited haematology and were referred to liver clinic for the evaluation of suspected portal hypertension after the haematological evaluation for anemia was found to be negative.

The next common presentation was variceal bleed ,seen in 43%(20) of the patients followed by ascites which was first presentation in 7%(3) of the patients(FIG 1) .

Hypersplenism as defined as cytopenia involving one or more cell lines in the setting of portal hypertension was present in 36 patients(78%).

FIG 1



Liver function tests showed mild unconjugated hyper bilirubinemia in 22 patients(48%) and Serum albumin was <3 in 10(7%) patients. Prothrombin time (PT) was near normal in all the patients with NCIPH. Median MELD score at presentation was 9 (Range 6-16).

Hepatic vein pressure studies:

Of the 46 NCIPH patients 39 underwent transjugular liver biopsies and HVPG was measure in 26 patients.

Of the 26 NCIPH patients who underwent pressure studies

- HVPG<5mm Hg was seen in 8 patients
- HVPG(5-10mm Hg) was seen in 11 patients
- HVPG>10mm Hg was seen in 7 patients.

Results of evaluation of folate metabolism:

NCIPH patients(n-46):

VITAMIN B12- Median levels of serum vitamin B12 levels was 323 pg/ml (Range 50-1976pg/ml) and levels <250 pg/ml was seen in 30% (14) of the patients as depicted in FIG 4.

SERUM FOLATE –Median serum folate level was 14.3 ng/ml (Range 1-22.6 ng/ml) and low folate levels(<3ng/ml) was seen in only one patient.

FASTING SERUM HOMOCYSTEINE-Fasting serum homocysteine could be measured in 19 NCIPH patients and the median value was 15.2 micromoles/l (Range 4.5-29.5 mic mol/l). Levels greater than 13 micromoles/l is considered as hyperhomocysteinemia [19] which was seen in 12 patients.

Healthy controls(n-109):

Of the 109 age and geography matched healthy controls included in the study, serum vitamin B12 and folate levels could be measured in 72 patients.

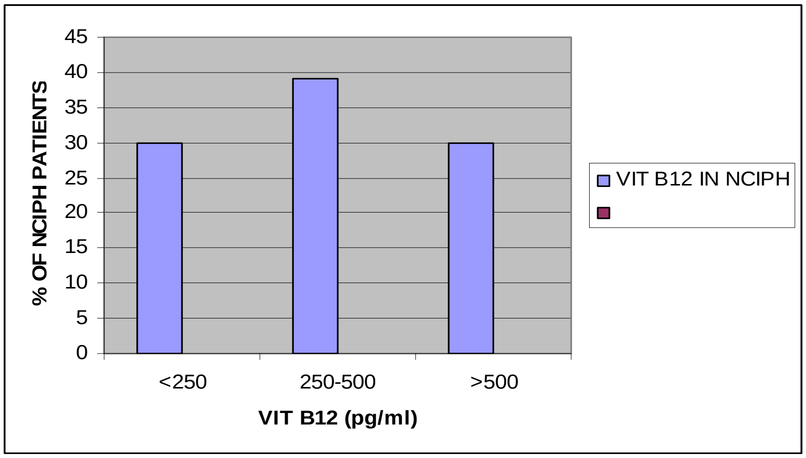
VIT B12-Median levels of serum vitamin B12 levels in matched healthy controls was 309.45pg/ml(Range 82.48-1027 pg/ml) and levels <250 pg/ml was seen in 36%(26) as depicted in FIG 5 and Table 2 .

SERUM FOLATE-Median serum folate level was 7.32ng/ml (Range 2-15.61 ng/ml) and low folate levels (<3ng/ml) was seen in two patients.

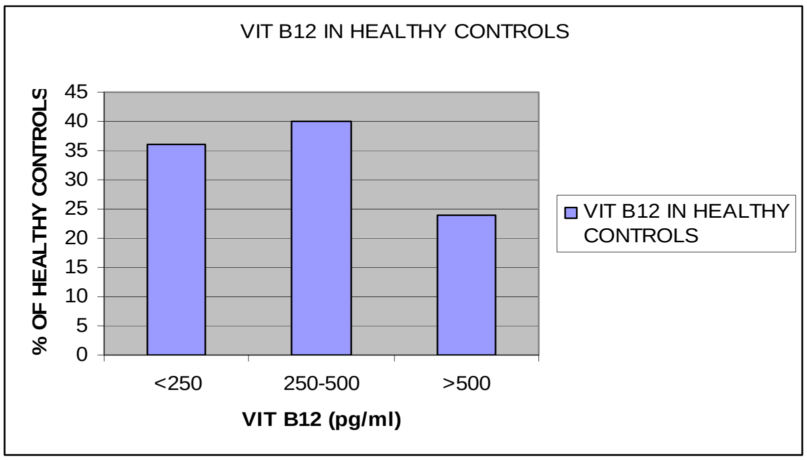
Cirrhosis (HBV/HCV)(n-42)-

VITAMIN B12-Median levels of serum vitamin B12 levels in matched cirrhosis patients was 1022.4 pg/ml(Range 171-2000 pg/ml) and levels <250 pg/ml was seen in 5% (2) of the patients as depicted in FIG 6 and Table 2 .

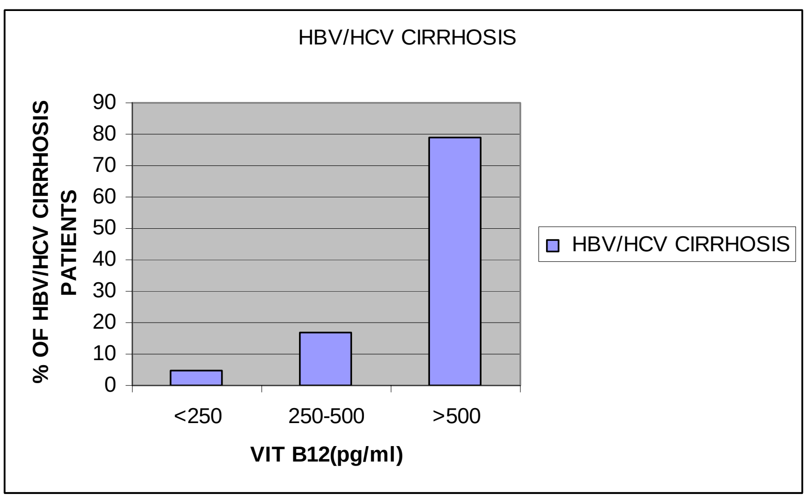
SERUM FOLATE-Median serum folate level was 9.58 ng/ml(Range 3.8-24 ng/ml) and low folate was found in none of the cirrhosis patients (Table 2).



% OF NCIPH PATIENTS
45
40
35
30
25
20
15
10
5
0
<250
250-500
>500
VIT B12 (pg/ml)
VIT B12 IN NCIPH



VIT B12 IN HEALTHY CONTROLS
% OF HEALTHY CONTROLS
45
40
35
30
25
20
15
10
5
0
<250
250-500
>500
VIT B12 (pg/ml)
VIT B12 IN HEALTHY CONTROLS



HBV/HCV CIRRHOSIS
% OF HBV/HCV CIRRHOSIS PATIENTS
90
80
70
60
50
40
30
20
10
0
<250
250-500
>500
VIT B12(pg/ml)
HBV/HCV CIRRHOSIS

The prevalence of vitamin B12 deficiency was significantly higher in NCIPH patients and healthy controls when compared to HBV/HCV cirrhosis (by Chi square test, P value being 0.002). There was no significant difference in the prevalence of folate deficiency in the three groups.

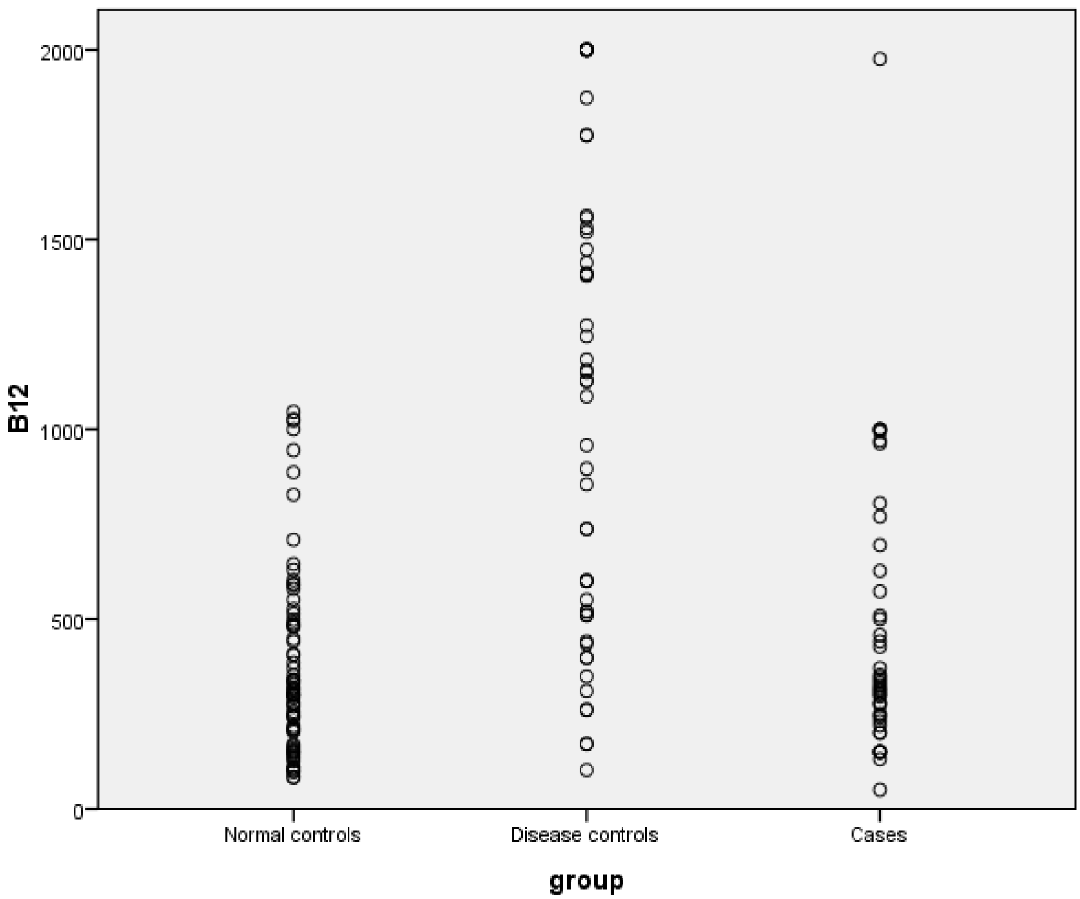
TABLE 2

SERUM VITAMIN B12 LEVELS	>250pg/ml	<250pg/ml(DEFICIENT)
NCIPH(N-46)	32	14
HEALTHY CONTROLS(N-72)	36	26
HBV/HCV CIRRHOSIS(N-42)	40	2

TABLE 3

SERUM FOLATE LEVELS	>3ng/ml	<3ng/ml(DEFICIENT)
NCIPH(N-46)	45	1
HEALTHY CONTROLS(N-72)	70	2
HBV/HCV CIRRHOSIS(N-42)	42	Nil

FIG 2-DISTRIBUTION OF VITAMIN B12 LEVELS IN THE THREE GROUPS-DEPICTION IN THE SCATTER PLOT



Vitamin B_{12} deficiency etiological workup

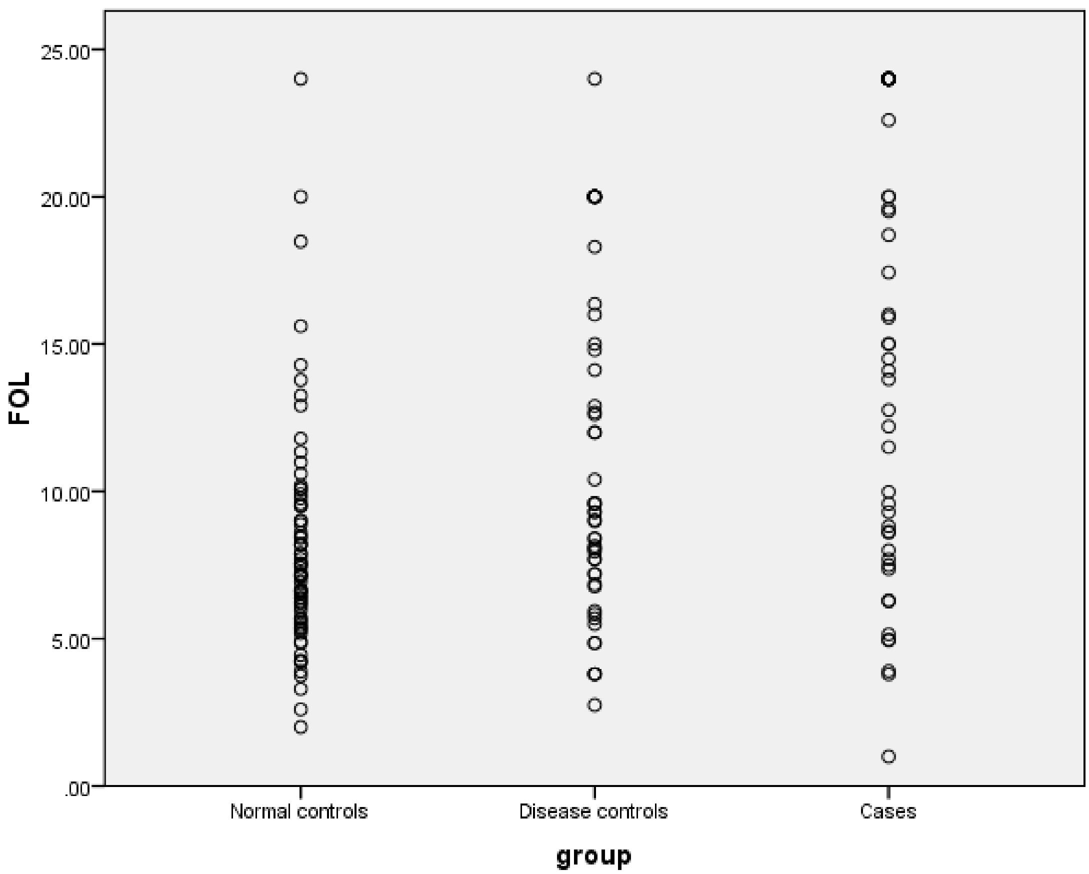
Complete etiological workup for vitamin B12 deficiency was not done in the present study.

Out of 13 NCIPH patients with vitamin B12 deficiency, 2 patients are pure vegetarians, 5 patients underwent duodenal biopsy of which 1 had villous atrophy, 1 had chronic duodenitis and 3 were normal.

The one with villous atrophy had low urine D xylose and 72 HR stool fat was high

IF (Intrinsic factor) antibody screen was done in 3 and all were negative for the antibody.

FIG 3-DISTRIBUTION OF FOLATE LEVELS IN THE THREE GROUPS-DEPICTION IN THE SCATTER PLOT



MCV and Iron deficiency in NCIPH patients

TABLE 4

NCIPH PATIENTS	MCV(fl) MEDIAN (RANGE)	IRON DEFICIENCY* (NUMBER OF PATIENTS)
VIT B12<250pg/ml#	84.2(54.2-100.1)	7
VIT B12>250pg/ml§	82.35(60-112)	13

*Serum Ferritin<20microgram/l in males and < 5 mic g/l in females and / or Transferrin saturation<18%

11 out of the total 13 NCIPH patients with vitamin B12 deficiency had iron indices tested

§ 21 out of the 33 NCIPH patients with normal vitamin B12 had iron indices tested

Of the 31 patients with NCIPH in whom iron indices were tested, evidence of iron deficiency was seen in 20 patients of which 7 had concomitant iron and vitamin B12 deficiency.

In vitamin B12 deficient NCIPH group, MCV was normal (70-90 fl) in 6, low(<70 fl) in 3 and high(>90fl) in 4 patients. Concomitant iron deficiency was seen in 7 patients of which 2 had elevated MCV, 2 had low MCV and 3 had normal MCV.

CORRELATION BETWEEN VITAMIN B12 AND SERUM HOMO CYSTEINE IN NCIPH PATIENTS-

Excluding the one outlier depicted in the graph(Fig 7) there was no significant correlation between serum VIT B12 and fasting serum homocysteine in NCIPH patients (by Spearman's correlation)

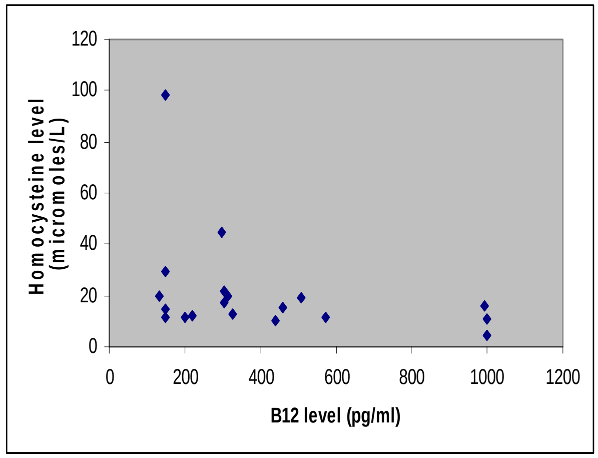


Fig 7

Correlation coefficient- 0.453 P value-0.052

CORRELATION BETWEEN SERUM VITAMIN B12 AND MELD IN NCIPH PATIENTS-

There was no significant correlation (Fig 8) between serum VITAMIN B12 and MELD in NCIPH patients (Spearman's correlation)

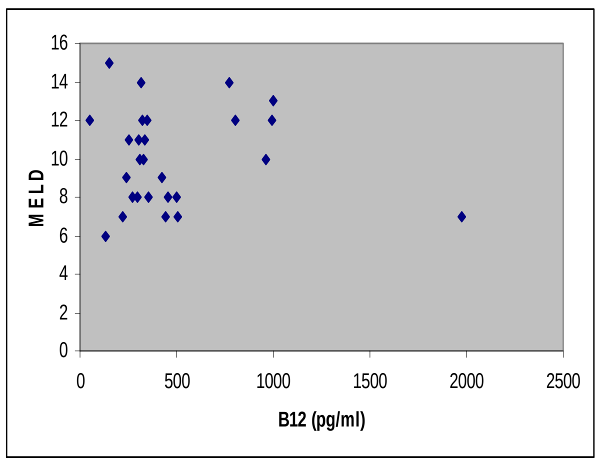


Fig 8

Correlation coefficient: 0.038 P value: 0.851

CORRELATION BETWEEN SERUM VITAMIN B12 AND MELD IN HBV/HCV CIRRHOSIS

Significant positive correlation(Fig 9) was seen between serum VITAMIN B12 and MELD in HBV/HCV cirrhosis.

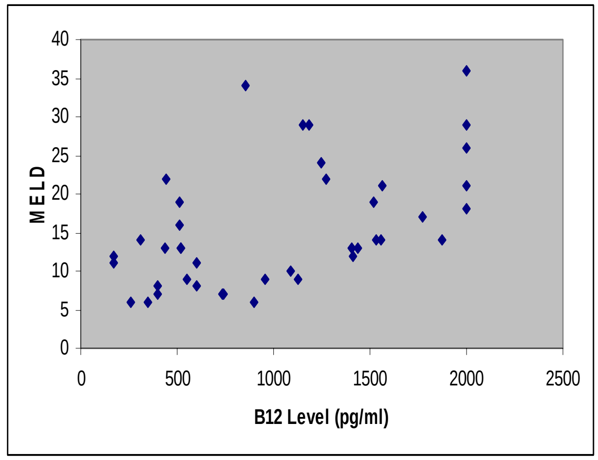


Fig 9

Correlation coefficient :0.55 P value:0.00

ANALYSIS OF MTHFR GENE POLYMORPHISM:

The two MTHFR gene polymorphisms C677T and A1298C were analysed by PCR- RFLP in three groups namely the NCIPH cases, healthy controls and cirrhotic(HBV/HCV) patients.

Healthy controls and Healthy controls along with Cirrhosis group followed the Hardy Weinberg equilibrium. (For which polymorphism?)

PREVALENCE OF MUTANT ALLELES FOR C677T POLYMORPHISM-

The prevalence of mutant alleles (T) in NCIPH group was 4%, healthy controls was 15% and in cirrhosis group was 13%.

Thus, the mutant alleles were significantly higher in Healthy controls when compared to NCIPH group with a P value of 0.02 (Fisher's exact test), Relative risk of 0.80(95% CI 0.71-0.91) and Odds ratio of 0.25(95%CI 0.07-0.84) were noted, indicating that the mutation had protective association against NCIPH with an effect of approximately 20%.

PREVALENCE OF C677T ALLELES IN HEALTHY CONTROLS AND NCIPH

ALLELES	H.CONTROLS	NCIPH	TOTAL
C677T			
C	185	67	252
T	33	3	36
TOTAL	218	70	288

P VALUE:0.02

PREVALENCE OF C677T ALLELES IN NCIPH AND HBV/HCV CIRRHOSIS

ALLELES	NCIPH	HBV/HCV CIRRHOSIS	TOTAL
C677T			
C	67	49	116
T	3	7	10
TOTAL	70	56	126

P VALUE:0.1

PREVALENCE OF MUTANT GENOTYPE FOR C677T POLYMORPHISM-

The prevalence of mutant genotype(heterozygous CT/homozygous TT) in NCIPH was 9%, healthy controls was 28% and Cirrhosis patients was 21% (sample size in the cirrhosis group was 28).

The mutant genotype was significantly higher in healthy controls when compared to NCIPH group with a P value of 0.02 (Fisher's exact test), Relative risk of 0.78(95% CI 0.67-0.92) and Odds ratio of 0.25(95%CI 0.07-0.86).

PREVALENCE OF C677T MUTANT GENOTYPES IN HEALTHY CONTROLS AND NCIPH

GENOTYPE	H.CONTROLS	NCIPH	TOTAL
WILD	79	32	111
MUTANT	30	3	33
TOTAL	109	35	144

P VALUE:0.02

PREVALENCE OF C677T MUTANT GENOTYPES IN NCIPH AND HBV/HCV CIRRHOSIS

GENOTYPE	NCIPH	HBV/HCV CIRRHOSIS	TOTAL
WILD	32	22	54
MUTANT	3	6	9
TOTAL	35	28	63

P VALUE:0.17

PREVALENCE OF MUTANT ALLELES FOR A1298C POLYMORPHISM-

The prevalence of mutant alleles(C) in NCIPH group was 34%,healthy controls was 40% and in cirrhosis group was 38%.

There was no statistically significant difference of the prevalence of mutant alleles between NCIPH group and healthy controls ; NCIPH group and cirrhosis group (Fisher exact test).

PREVALENCE OF A1298C ALLELES IN HEALTHY CONTROLS AND NCIPH

ALLELES	N.CONTROLS	NCIPH	TOTAL
A1298C			
A	129	46	175
C	85	24	109
TOTAL	214	70	284

P VALUE-0.47

PREVALENCE OF A1298C ALLELES IN NCIPH AND HBV/HCV CIRRHOSIS

ALLELES	NCIPH	HBV/HCV CIRRHOSIS	TOTAL
A1298C			
A	46	37	83
C	24	19	43
TOTAL	70	56	126

P VALUE-1

PREVALENCE OF MUTANT GENOTYPES FOR A1298C POLYMORPHISM:-

The prevalence of mutant genotypes(Heterozygous AC/Homozygous CC) in NCIPH group was 57%, healthy controls was 70% and in cirrhosis group was 46%.

There was no statistically significant difference of the prevalence of mutant genotypes between NCIPH group and healthy controls ; NCIPH group and cirrhosis group(Fisher exact test).

PREVALENCE OF A1298C MUTANT GENOTYPES IN HEALTHY CONTROLS AND NCIPH

GENOTYPE	H.CONTROLS	NCIPH	TOTAL
WILD	32	15	47
MUTANT	75	20	95
TOTAL	107	35	142

P VALUE-0.21

PREVALENCE OF A1298C MUTANT GENOTYPES IN NCIPH AND HBV/HCV CIRRHOSIS

GENOTYPE	NCIPH	HBV/HCV CIRRHOSIS	TOTAL
WILD	15	15	30
MUTANT	20	13	33
TOTAL	35	28	63

P VALUE-0.45

DISCUSSION

The current study is a prospective evaluation of folate metabolism with particular reference to MTHFR gene mutation (C677T nad A1298C polymorphisms) in Idiopathic Non cirrhotic Intrahepatic Portal Hypertension(NCIPH).

DEMOGRAPHY:

The median age at presentation in NCIPH was 32.6 yrs(Range 15-62). Several studies in the past described mean age ranging from 25-35 years[29]. Koshy described a double peak in age incidence one at 21-25 yrs and the other at 36-40 yrs[49].

The male to female ratio was 2.13:1 as was seen in series from different parts of India(2:1 to 4:1)[12,16].Majority of patients were from North and Northeastern India..

CLINICAL AND LAB FEATURES IN IDIOPATHIC NCIPH:

In our study, the most common first clinical presentation was anemia with lump in the abdomen which was seen in 23 patients(50%) followed by variceal bleed seen in 43%. Ascites was the presentation in 3 patients . In a data based experience from North India, variceal bleed was seen in 84%, LUQ mass in 13.5%and ascites in 10% in NCPF[29].In another retrospective study from North India lump in the abdomen was the commonest first presentation (68.9%) followed by gastrointestinal bleeding[5].

Of the patients presenting with anemia and lump(splenomegaly),17 went through haematology OPD and after exclusion of the primary haematologic disorder were referred to Liver clinic.

78% of the patients had hypersplenism defined as cytopenia involving one or more cell lines in the setting of portal hypertension which is similar to that seen in other studies[5].

LFT showed mild unconjugated hyperbilirubinemia in 22 patients (48%) and serum albumin < 3 gm% in 10 patients though Prothrombin time (PT) was near normal in all. The parenchymal damage manifest by increased aminotransferase levels is very minimal in NCPF. Results of conventional tests of liver function are normal or near normal[5]. Progression to liver failure is increasingly documented in NCIPH in a subset of patients [8, 50].

Hillaire et al in their study[8] documented serum Bilirubin as high as 5.4mg% at initial presentation.One of the patient in our study group underwent liver transplantation due to liver failure(in the form of recurrent hepatic encephalopathy) , the explant biopsies showed no evidence of Cirrhosis but had histological features consistent with NCIPH[1].

HEPATIC VEIN PRESSURE STUDIES:

Of the 46 NCIPH patients ,39 had Transjugular liver biopsies(indication being coagulopathy in the form of thrombocytopenia). HVPG was measured in 26 patients of which only 8 had HVPG <5mm Hg and rest had HVPG>5mm Hg. Elevated HVPG has been reported in NCIPH, probably reflecting peri-sinusoidal resistance in addition to pre-sinusoidal resistance to portal venous blood flow[51].

SIGNIFICANT DIFFERENCES IN VITAMIN B12 DEFICIENCY IN THE 3 STUDY GROUPS:

Serum vitamin B 12 levels < 250 pg/ml was seen in 30% of NCIPH group and 36% of age geography matched healthy controls.In patients with HBV/HCV cirrhosis , vitamin B12 deficiency was seen in only 5% and median levels were 1022.4 pg/ml(Range 171-2000 pg/ml).

WHY IS VITAMIN B12 DEFICIENCY SEEN IN 30% OF NCIPH PATIENTS?

High levels of serum vitamin B12 in cirrhosis patients was probably due to

hepatic parenchymal injury .Vitamin B12 is stored in the liver (estimated 1 mg) and serum levels of vitamin B12 as assayed biologically are present in a variety of liver disorders particularly those accompanied by necrosis of liver cells [52].The total serum content based on an average serum serum concentration of 300 ng/ml is of the same order as the total requirements estimated at about 1 mic g a day(This is the minimum dosage necessary to maintain a patient with pernicious anemia in haematologic remission) .

NCIPH is a vascular disorder affecting hepatic vasculature at the presinusoidal level without hepatocyte injury and hence the median vitamin B12 levels were significantly low when compared to Cirrhosis group and prevalence of vitamin B12 deficiency was almost similar to that of Healthy controls.

HOMOCYSTEINE AND VIT B12 DEFICIENCY IN NCIPH:

Fasting serum Homocysteine levels were elevated(>13micr mol/l) in 12 out of the 19 NCIPH patients in whom it was tested.The median levels were 15.2 micromoles/l(Range 4.5-98 mic mol/l). There was no significant correlation between vitamin B12 and fasting serum Homocysteine levels. This may be because it is known fact that relative deficiency of vitamin B12 or folate cause elevation in serum homocysteine levels and most of the patients had been to various hospitals before coming to liver clinic and had been on vitamin supplements.

There was no significant difference between the serum folate levels in the three groups studied (NCIPH,Healthy controls and Cirrhosis group).

Prevalence of vitamin B12 deficiency in NCIPH in our study was nearly the same as seen in healthy controls .However significant difference was seen in HBV/HCV cirrhosis(majority had elevated vitamin B12 and only 2 patients had low levels).

We thought of two explanations for this finding:

1. Hepatocyte injury leading to the release of vitamin B12 into the circulation in HBV/HCV cirrhosis.
2. Vitamin B12 deficiency is causally linked to NCIPH

<u>B12 DEFICIENCY CAUSALLY LINKED TO NCIPH:</u>

Our original hypothesis was that vitamin B12 being a gut derived vitamin, B12 deficiency would be maximal in the portal system. This could be a prothrombotic factor driving microvascular occlusion in portal vascular tree.

Vitamin B12 deficiency was significantly higher compared to HBV/HCV cirrhosis in our study.However ,the lack of significant difference in vit B12 deficiency amongst NCIPH patients and healthy controls appears not to fit our original hypothesis.

One possibility is that vitamin B12 deficiency is so common in our population (about a third of healthy controls studied), this makes it difficult to show causal association between B12 deficiency and NCIPH. We have not studied the causes of vitamin B12 deficiency in detail in our study.

We postulate that silent gut disorders like tropical enteropathy may be involved. In support of this, of the 5 patients who underwent duodenal biopsies ,one had villous atrophy ,another reported as chronic duodenitis and the rest 3 had no significant lesion .

MTHFR C677T & A1298C POLYMORPHISMS:

The two MTHFR gene polymorphisms C677T and A1298C were analysed in the three groups .

The prevalence of mutant alleles(T) in NCIPH group was 4%,Healthy controls was 15% and the prevalence of mutant genotype(heterozygous CT/homozygous TT) in NCIPH was 9%,healthy controls was 28%.

The mutant genotype and the mutant alleles were significantly higher in Healthy controls when compared to NCIPH group with a P value of 0.02 (Fisher's exact test).

There was no statistically significant difference of the prevalence of mutant genotypes and mutant alleles for A1298C polymorphism between NCIPH group and healthy controls ; NCIPH group and cirrhosis group.

In a study published from Turkey[28] where they analysed the inherited thrombophilic mutations and natural anticoagulant deficiency in patients with idiopathic portal hypertension,_MTHFR C677T and MTHFR A1298C frequencies of genetic polymorphisms were found to be significantly higher among patients than that of controls.But the sample size in this study was too small with 12 NCIPH and 13 controls.

In contrast, in our study we found MTHFR C677T frequencies of genetic polymorphisms significantly higher in healthy controls than NCIPH group and there was no signicant difference for A1298 Cpolymorphism.

Our data calls for further studies in larger number of patients.

IS VITAMIN B12 DEFICIENCY OVERLOOKED IN NCIPH?

We found that of the 14 patients with low vitamin B12 ,MCV was elevated in 4.

It is possible that concomitant iron deficiency accounts for the normal/low MCV in the rest of the patients.

Our study suggests that serum vitamin B12 levels should be routinely tested in Idiopathic Non cirrhotic Intrahepatic portal hypertension(NCIPH).

CONCLUSIONS

1. The median serum level of vitamin B12 was significantly higher in patients with cirrhosis when compared with Idiopathic Non cirrhotic Portal hypertension(NCIPH) and the prevalence of vit B12 deficiency was 30% in NCIPH compared to 36% in healthy controls(36%).

   Inference-This reflects the parenchymal damage in cirrhosis causing release of vit B12 into the circulation and that there is no parenchymal(hepatocyte) injury in NCIPH. (Reference??)

2. Among the NCIPH patients with low vitamin B12, MCV was low or normal in the majority with concomitant iron deficiency seen in the majority of the patients (7 out of 11 tested).

3. Fasting serum Homocysteine was elevated in 12 out the 19 patients of NCIPH in whom it was tested ,but there was no correlation with vitamin B12 deficiency.

4. There was no difference in serum folate levels in the NCIPH group when compared to Cirrhosis patients or Healthy controls.

5. MTHFR C677T Polymorphism (both mutant alleles and mutant genotypes)was more prevalent in healthy controls than NCIPH with a P value of 0.02 (Fisher's exact test),Relative risk of 0.78(95% CI 0.67-0.92) and Odd's ratio of 0.25(95%CI 0.07-0.86).

Inference: MTHFR C677T polymorphism being significantly more common in healthy population than NCIPH patients in our study suggests either a protective mutation or a chance association. This requires further study.

6. There was no difference in the prevalence of A1298C MTHFR poly morphism between NCIPH patients and healthy controls.

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PROFORMA

Serial Number

Name

Age

Sex

Hosp.Number

Address

First clinical presentation

HISTORY OF

Gastrointestinal bleed

Abdominal distension

Altered sensorium

Mucosal bleed (Gum bleed / Epitaxis etc)

Personal and Dietary history

CLINICAL EXAMINATION

LAB INVESTIGATION

CBC

LFT

PT

Etiological workup for known causes of liver disease

MELD score at presentation

IMAGING

Ultrasound abdomen / Colour Doppler

GASTROSCOPY

LIVER BIOPSY

HVPG (Hepatic venous pressure gradient)

SERUM VITAMIN B12 / SERUM FOLATE

FASTING SERUM HOMOCYSTEINE

MTHFR GENE MUTATION ANALYSIS

CONSENT FORM

I understand that Dr. K.MADHU is doing a study to evaluate the causative factors in patients with non-cirrhotic portal hypertension and cirrhosis of unknown cause. The studies involve blood tests to detect how my body utilizes a vitamin that is necessary for good health of the blood vessels. The results of the test done in connection with the study may directly benefit me. They are likely to indirectly benefit other patients with the disease.

I accept to undergo the work up required to be done during the study and provide the blood samples for estimation of serum vitamin B12, Folate and homocystein levels and MTHFR gene mutation.

I understand that my withdrawal from the study, at any time will not affect the treatment being given.

Study Title:

Study Number:

Subject's Initials: __________ Subject's Name: _________

Date of Birth / Age:________

Please initial box

(Subject)

(i) I confirm that I have read and understood the information sheet dated _________ for the above study and have had the opportunity to ask questions. [ ]

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

(iii) I understand that the Sponsor of the clinical trial, others working on the Sponsor's behalf, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s) [ ]

(v) I agree to take part in the above study. [ ]

Signature (or Thumb impression) of the Subject/Legally Acceptable Representative:_____________

Date: _____/_____/______

Signatory’s Name: ___________________________________

Signature of the Investigator: _________________________

Date: _____/_____/______

Study Investigator’s Name: __________________________

Signature of the Witness: ____________________________

Date:_____/_____/_______

Name of the Witness: _______________________________